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Scored Antibody Reactivity Determined by Immunoblotting Shows an Association between Clinical Manifestations and Presence of *Borrelia burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. Valaisiana* in Humans

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An immunoglobulin G immunoblot was developed with antigenic extracts of *Borrelia burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. valaisiana* genospecies and was reacted with sera from patients with neuroborreliosis, acrodermatitis, and Lyme arthritis. A detailed analysis of the reactivities of the protein bands was performed, and a two-step scoring procedure was selected to determine the preferential reactivity of sera to one particular genospecies. The discriminative potential of 5 proteins (12-kDa, 16-kDa, 18-kDa, OspA, and 66-kDa proteins) was used as a rapid first-step scoring method, followed by scoring of 14 additional protein bands if necessary. The advantage of this procedure is the low percentage of serum samples with inconclusive results for one of the four species (10% for patients with neuroborreliosis, 6% for patients with acrodermatitis chronica atrophicans, and 6% for patients with Lyme arthritis). Among 31 serum samples from patients with neuroborreliosis, 16 were more reactive to *B. garinii*, 7 were more reactive to *B. afzelii*, 3 were more reactive to *B. valaisiana*, and 2 were more reactive to *B. burgdorferi* sensu stricto. Of 31 serum samples from patients with acrodermatitis, 26 showed a higher level of reactivity to *B. afzelii*. Of 34 serum samples from patients with Lyme arthritis, 21 were more reactive to *B. burgdorferi* sensu stricto, 10 were more reactive to *B. afzelii*, and 1 was more reactive to *B. valaisiana*. Our results suggest an organotropism of *Borrelia* species and provide some evidence of a pathogenic potential of *B. valaisiana* in humans.

Lyme borreliosis (LB) is a tick-borne multistage disease caused by the spirochetal bacterium *Borrelia burgdorferi* sensu lato. *B. burgdorferi* sensu lato is divided into several species on the basis of phenotypic and genotypic characteristics (35). In Europe, five species of *B. burgdorferi* sensu lato have been isolated from *Ixodes ricinus*: *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii* (4, 9), group VS116, which has been classified as *B. valaisiana* novel species (44), and *B. lusitaniae* (24).

It appears that the geographic distributions of these species are not uniform, even within neighborhoods (34). However, in Western Europe and Switzerland, *B. garinii* is more frequently isolated, followed by *B. afzelii*, *B. burgdorferi* sensu stricto, and *B. valaisiana*, in that order (39). In Scandinavia and The Netherlands, *B. afzelii* is probably the most common *Borrelia* (36, 42), followed by *B. valaisiana*, *B. burgdorferi* sensu stricto, and *B. garinii*. In Ireland, *B. valaisiana* is described as the most prevalent genospecies, followed by *B. garinii*, *B. burgdorferi* sensu stricto, and *B. afzelii* (23). *B. lusitaniae* was first isolated from *I. ricinus* in Portugal and was subsequently isolated from ticks in other European countries (24).

After a person is bitten by an infected tick, the spirochetes undergo a hematogenous dissemination and can be found in many of the major organ systems. The first stage and hallmark of LB is a distinctive skin rash, erythema migrans (EM), that frequently appears at the site of the tick bite (40). Days to months after the tick bite, the disease may progress toward secondary and tertiary stages. In some patients, chronic diseases may develop. These may affect the skin, such as acrodermatitis chronica atrophicans (ACA), a clinical manifestation primarily observed in Europe, and possibly affect joints with arthritis, which is more common in the United States (40). In Europe, neurological symptoms appear in 30% of untreated patients and musculoskeletal symptoms appear in 20% of patients (1). These observations have suggested that clinical outcome could depend on infection with strains of different genospecies. Neurological symptoms are thought to be mainly caused by *B. garinii*, while *B. afzelii* is often associated with ACA and *B. burgdorferi* sensu stricto is often associated with Lyme arthritis (3, 13, 32, 42). However, controversial reports described a good match between the distribution of *B. burgdorferi*: sensu lato in ticks and in patients from the same area (14) or an organotropism linked to strain-specific characteristics, not to genotypes (45). Furthermore, the pathogenic potential of *B. valaisiana* was suggested among patients with EM by using PCR (37), but there is no previous indication of an association of *B. valaisiana* with chronic clinical symptoms.

Immunoblotting is unanimously reported to be a confirmatory test for LB. One of the factors affecting the performance of this assay is the polymorphism of *Borrelia* antigens which is evident among species and intraspecies. Norman et al. (30) found that the preferential reactivity to genospecies is not absolute and that regional variations in the reactivity to the genospecies strains may occur. The purpose of the current study was to compare the immunoglobulin G (IgG) immunoblots of four different *Borrelia* genospecies present in Switzerland (34). Therefore, we tested sera from patients living in Switzerland. In order to decrease the percentage of serum samples with an inconclusive predominance of one of the four species, we modified the scoring method described by Péter et

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al. (32). The preferential reactivity of sera led us to confirm the association between some manifestations of LB and the species of *B. burgdorferi* sensu lato.

(This research is part of the Ph.D dissertation of K. Ryffel.)

MATERIALS AND METHODS

Study samples. Serum samples from Swiss patients with a clinical diagnosis of late LB and a positive screening test confirmed by immunoblotting were collected among sera referred for testing by a confirmatory diagnostic procedure. The sera were from 31 patients with neuroborreliosis, 31 patients with ACA, and 34 patients with Lyme arthritis. Sera from patients with several symptoms of LB were excluded. Among serum samples from patients with neuroborreliosis, all were confirmed to be positive by intrathecal antibody synthesis. The index of intrathecal antibody production was calculated as follows: IgG-specific titer in cerebrospinal fluid (CSF)/IgG-specific titer in serum divided by albumin concentration in CSF/albumin concentration in serum (negative result, index below 2.0).

In order to establish the persistence of the reactivity to the infecting species after antibiotic therapy, parallel serum samples from patients were tested. Patients with neuroborreliosis ($n = 2$), ACA ($n = 6$), and arthritis ($n = 1$) were selected. The first serum samples were taken during the first clinical visit, and the second ones were taken 6 months to 5 years after treatment.

Antigen preparation. *Borrelia* strains, *B. burgdorferi* sensu stricto VS215, *B. garinii* VS102, *B. afzelii* VS461, and *B. valaisiana* VS116 were used for antigen preparation (32, 44). All strains were isolated from ticks (*I. ricinus*) (33). Spirochetes were cultured in BSK II medium (Sigma, St. Louis, Mo.). During the late logarithmic phase of growth, the culture was centrifuged at $14,000 \times g$ for 15 min and washed twice in phosphate-buffered saline (pH 7.2) to which $MgCl_2$ (0.05 M) was added. The protein concentration of the suspension was determined by the biuret method and was adjusted to 1 mg/ml in distilled water. Washed *Borrelia* cells were stored at −20°C until use (34). The isolates used in this study were all low-passage strains. Prior to Western blotting, all antigen preparations were adjusted to contain equal amounts of p41, which is known to be present in rather constant amounts in all strains (16, 50) and which was quantified by serial dilution by Coomassie blue-stained immunoblotting and a densitometry analysis program.

Electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed by standard procedures (33). Briefly, samples were heated for 5 min at 95°C before undergoing electrophoresis on a polyacrylamide gel at 12.5% (constant voltage, 170 V). After electrophoresis the proteins were transferred to a polyvinylidene difluoride membrane and were cut into strips.

Before use, the strips were blocked for 1 h at 37°C with Tris-buffered saline (TBS; pH 7.2) with 5% gelatin and were washed three times for 5 min each time with washing buffer (W-buffer; TBS with 0.1% gelatin and 0.05% Tween 20) at room temperature. Each step of this procedure was performed at room temperature. The four antigen strips were incubated for 2 h with patient serum diluted 1/500 in D-buffer (TBS with 1% gelatin and 0.05% Tween 20). The strips were washed three times for 5 min each time with W-buffer. After the washing, rabbit anti-human IgG conjugated to alkaline phosphatase diluted 1/1,000 in D-buffer was added. At the end of the second 2-h incubation, two washes were done with W-buffer and one was done with TBS. The bound conjugate was visualized by addition of the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate–Nitro Blue Tetrazolium (Kirkegaard & Perry Laboratories). The reaction was stopped 10 to 15 min later by two rinses in distilled water. Incubations were always performed with all the strips of each antigen in the same well.

Characterization of protein bands. Reproducibility was confirmed by repeat probing of the immunoblot strips from different gels with four serum samples preferentially reactive with each species. In all strains the 39-kDa protein could be clearly differentiated from the flagellin (41 kDa), the 30-kDa protein could be differentiated from OspA, and the 58-kDa protein could be differentiated from the 60-kDa protein.

The blots were analyzed visually by one person, and the interpretation was confirmed independently by an other one. Since only minor divergences were observed for the typing of the serological reactions between the two readers, we used the classification of one reader only. For comparison between the four strips, scores (0 to 3 points) were allocated depending on the presence and intensity of the reaction to 19 proteins of *Borrelia*: the 93-kDa (p100), 75-kDa, 66-kDa, 60-kDa, 58-kDa, 54- to 56-kDa, 45-kDa, 41-kDa (flagellin), p39, 36-kDa, OspA, 30-kDa, OspD, OspC, 20-kDa, 18-kDa, 16-kDa, 14-kDa, and 12-kDa proteins. Some of these proteins were identified in the four *Borrelia* species with monoclonal antibodies: LA 114 Zs7 (93-kDa protein) sl60 (60-kDa protein), H9724 (flagellin), LA 112 Zs7 (39-kDa protein), and LA 22 2B8 (OspC) (kindly provided by A. G. Barbour, University of California, Irvine; R. Wallich, Ruprecht-Karls-Universität, Heidelberg, Germany; and B. Wilske, Max von Pettenkofer Institut, Munich, Germany). OspA was identified with two different monoclonal antibodies, H5332 (reactive with VS215, VS102, and VS461) and A116k (reactive with *B. valaisiana*). A report characterizing the A116k monoclonal antibody is in preparation. The other proteins were identified in the four *Borrelia* species by specific serum reactivities and Molecular Analysis software (Bio-Rad, Hercules, Calif.) (see Fig. 1 and Table 1).

Determination of predominant reactivity for each species. To determine the preferential reactivities of the sera, a detailed analysis was performed. Five of 13 proteins with a discriminative potential (the 12-kDa, 16-kDa, 18-kDa, OspA and 66-kDa proteins) were selected and were used in a rapid first-step scoring procedure (method I). A total score for these five proteins that was greater by two points for one individual species compared with the scores for the other species was considered as preferential reactivity. If preferential reactivity could not be found, the scores for all 19 protein bands were taken into account (method II). A total score that was three points greater for one individual species compared with the scores for the other species was considered a preferential reactivity. The differential number of scored points necessary to define this preferential reactivity to one particular species was established on the basis of a previous statistical study (32).

Comparison of immunoreactivity with genotypic detection. Oligonucleotide primers that recognize the OspA sequences of most *B. burgdorferi* strains (11) were used to amplify DNA by PCR for patients for whom there was a clinical suspicion of Lyme arthritis. The presence of *B. burgdorferi* sensu lato DNA in synovial fluid ($n = 15$) and urine ($n = 1$) samples was shown by agarose gel analysis. The specificities of the PCR amplicons were controlled by colorimetric solid-phase capture hybridization assay (28). Stored aliquots of each PCR-positive synovial fluid or urine sample were retrospectively analyzed with species-specific primers to type the infecting strains by PCR. (11). Without knowing the results of the PCR, sera from these patients were analyzed and the results were interpreted independently.

RESULTS

Characterization of serological responses. In order to determine the preferential reactivities of sera from patients with neuroborreliosis ($n = 31$), ACA ($n = 31$), and Lyme arthritis ($n = 34$), the bands for following proteins were scored and analyzed on four species-specific immunoblots: the 93-kDa (p100), 75-kDa, 66-kDa, 60-kDa, 58-kDa, 54- to 56-kDa, 45-kDa, 41-kDa (flagellin), p39, 36-kDa, OspA, 30-kDa, OspD, OspC, 20-kDa, 18-kDa, 16-kDa, 14-kDa, and 12-kDa proteins (Fig. 1; Table 1).

When the specimens were grouped by their preferential reactivity to each of the four species, only a few bands appeared to have discriminative potential. Among the sera with a preferential reactivity to *B. burgdorferi* sensu stricto ($n = 23$), the 12-kDa, 16-kDa, OspC, 58-kDa, and 66-kDa antigen bands were found to have a higher discriminative power with *B. burgdorferi* sensu stricto than with the other species. Sera with a preferential reactivity to *B. garinii* ($n = 17$) had greater reactivities to the 16-kDa, 18-kDa, 20-kDa, OspC, OspD, 30-kDa, OspA, 45-kDa, and 60-kDa antigen bands. Sera with a preferential reactivity to *B. afzelii* ($n = 43$) had greater reactivities to the 12-kDa, 14-kDa, 16-kDa, 18-kDa, OspC, and 45-kDa antigen bands. Among sera with a preferential reactivity to *B. valaisiana* ($n = 4$), the reactivities to the OspA, 45-kDa, and 66-kDa antigen bands were greater. The 12-kDa, 14-kDa, 16-kDa, 18-kDa, 20-kDa, OspC, OspD, 30-kDa, OspA, 45-kDa, 58-kDa, 60-kDa, and 66-kDa proteins had discriminative potential for the four species. On the basis of these results, the five proteins with the best discriminative potential (the 12-kDa, 16-kDa, 18-kDa, OspA, and 66-kDa proteins) were used in a rapid first-step procedure to determine the preferential reactivities of sera to the four species (Fig. 2A to D). If this procedure was not discriminative, the second step was then used and the results were determined on the basis of the scores for all 19 proteins.

Among the sera from patients with neuroborreliosis, 18 serum samples had a preferential reactivity to one species after the first step; the results for 16 of them were confirmed by the second step. For two samples the total score was not greater by three points. Among the sera from patients with ACA and Lyme arthritis, 21 and 19 serum samples, respectively, had a preferential reactivity to one species after the first step. The results for 19 and 17 of the samples respectively, were confirmed by the second step. For the four remaining serum samples the total score was not greater by three points for one

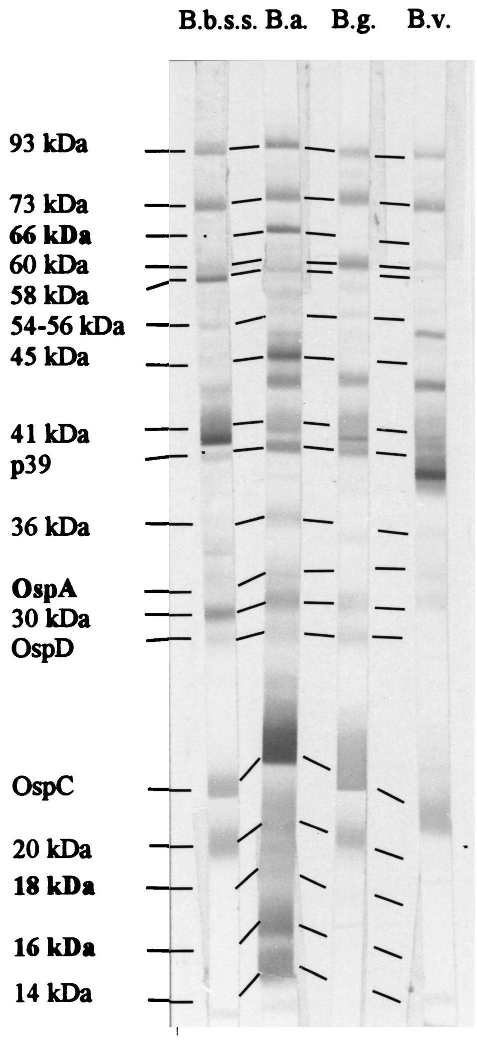

FIG. 1. Example of an immunoblot with a predominant score for *B. afzelii*. Immunoblots (IgG) were done with the serum from a patient with ACA. B.b.ss., *B. burgdorferi* sensu stricto VS215; B.a., *B. afzelii* VS461; B.g., *B. garinii* VS102; B.v., *B. valaisiana* VS116. Boldface indicates the five discriminative proteins used in method I.

TABLE 1. Scores for reactivity of a serum sample to the four *Borrelia* species for 19 proteins[a]

Protein band	Score[b]			
	B.b.ss	B.a.	B.g.	B.v.
93 kDa	2	2	1	1
73 kDa	2	2	2	2
66 kDa	**0**	**3**	**0**	**0**
60 kDa	1	0	2	1
58 kDa	2	1	0	1
54–56 kDa	1	1	1	0
45 kDa	1	3	0	0
41 kDa	2	2	2	2
p39	1	2	2	2
36 kDa	0	1	0	1
OspA	**1**	**1**	**0**	**1**
30 kDa	2	2	1	1
OspD	1	1	1	0
OspC	2	3	2	2
20 kDa	2	2	2	0
18 kDa	**0**	**1**	**0**	**0**
16 kDa	**0**	**2**	**0**	**0**
14 kDa	0	2	0	0
12 kDa	**0**	**0**	**0**	**0**
Score method I[c]	**1**	**7**	**0**	**1**
Score method II[d]	20	31	16	14

[a] See Fig. 1. Boldface proteins and scores indicate the five discriminative proteins used in method I and the scores obtained with those proteins.
[b] B.b.ss., *B. burgdorferi* sensu stricto; B.a., *B. afzelii*; B.g., *B. garinii*; B.v., *B. valaisiana*.
[c] Score with the five discriminative proteins (the score was discriminative if the score for reactivity to one species was two points greater than the scores for reactivity to the other species).
[d] Total score with 19 proteins (the score was discriminative if the score for reactivity to one species was three points greater than the scores for reactivity to the other species).

species than for the others. The percentages of serum samples with preferential reactivity to a particular species are shown in Table 2. The percentages of serum samples with a preferential reactivity by the combined method were 90% for patients with neuroborreliosis (28 of 31), 94% for patients with ACA (29 of 31), and 94% for patients with Lyme arthritis (32 of 34). Of the three serum samples from patients with neuroborreliosis with undetermined reactivities, one was from a patient with an early infection and had a weak reaction for IgG. We suspect a mixed infection for another sample, as the total scores for reactivity to the 19 bands were clearly higher with the *B. garinii* and *B. afzelii* species-specific immunoblots than those with the *B. valaisiana* and *B. burgdorferi* sensu stricto species-specific immunoblots. Of the two serum samples from patients with Lyme arthritis and inconclusive reactivities to a particular genospecies, one seemed to be from a patient with a mixed infection. The total scores for the five specific bands were much higher with the *B. burgdorferi* sensu stricto and *B. afzelii* species-specific immunoblots than with the *B. garinii* and *B. valaisiana* species-specific immunoblots.

Two protein bands presented a preferential reactivity to one specific species, regardless of the clinical symptoms. The 14-kDa protein of *B. afzelii* and the 58-kDa protein of *B. burgdorferi* sensu stricto had higher reactivities.

Preferential serological reactivities of sera from patients with different clinical symptoms. The percentages of serum samples from each patient group showing a preferential reactivity to each species are presented in Table 3. Reactivities of sera from patients with neuroborreliosis were higher with the *B. garinii*-specific immunoblot (52%) than with those specific for other species (6 to 22%). The reactivities of sera from patients with ACA were predominant with the *B. afzelii*-specific immunoblot (84%). The reactivities of sera from patients with Lyme arthritis were higher with the *B. burgdorferi* sensu stricto-specific immunoblot (62%) and with the *B. afzelii*-specific immunoblot (29%). Three serum samples from patients with neuroborreliosis and one serum sample from a patient with Lyme arthritis had preferential reactivities to *B. valaisiana*-specific immunoblot.

Persistence of reactivity to species-specific immunoblot. The persistence of the predominant reactivity after treatment with antibiotics was evaluated by comparing sequential serum samples from nine patients. Two paired serum samples from patients with neuroborreliosis were analyzed. Both serum samples from one of them had a preferential reactivity to *B. valaisiana*-specific immunoblot. For the other patient, the reactivity of the first serum sample was inconclusive and the second serum sample had a preferential reactivity to *B. afzelii*-specific immunoblot. A decreasing IgG antibody response was

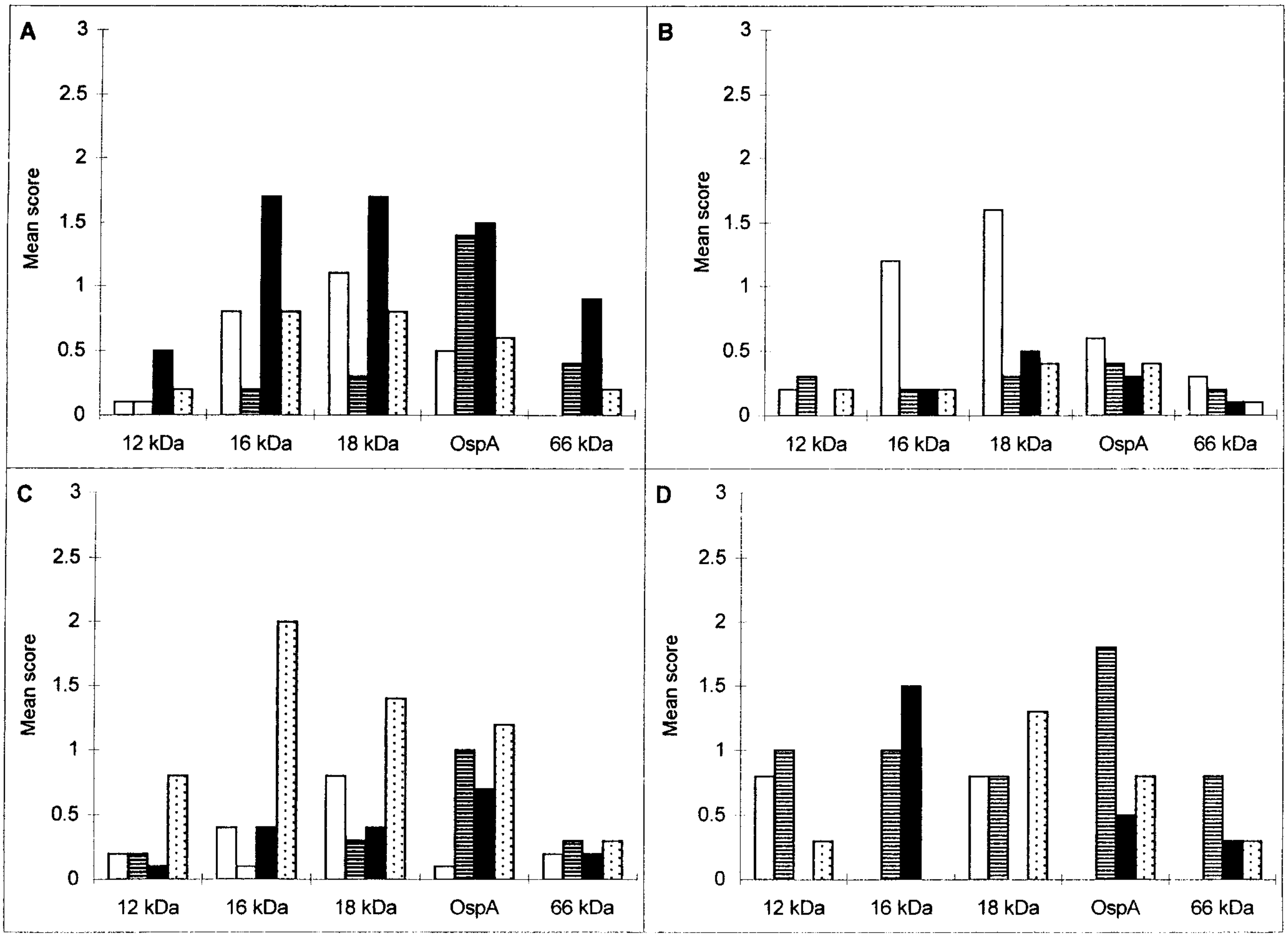

FIG. 2. Mean score of specific reactivity to different *Borrelia* species. (A) Specific reactivity to *B. burgdorferi* sensu stricto (n = 23). (B) Specific reactivity to *B. garinii* (n = 17). (C) Specific reactivity to *B. afzelii* (n = 43). (D) Specific reactivity to *B. valaisiana* (n = 4). ■, *B. burgdorferi* sensu stricto; □, *B. garinii*; ▨, *B. afzelii*; ▤, *B. valaisiana*.

observed for both of these patients. Six pairs of serum samples from patients with ACA were analyzed. For both serum samples from five of the patients, the sera had a preferential reactivity to *B. afzelii*-specific immunoblot and the second serum sample had a decreased immune response. The second serum sample from one of the patients had a preferential reactivity only to *B. burgdorferi* sensu stricto-specific immunoblot, although a decrease in the immune response was observed. One pair of serum samples from a patient with Lyme arthritis was analyzed. Both serum samples had preferential reactivity to *B. burgdorferi* sensu stricto-specific immunoblot, with an increased immune response for the second serum sample (obtained 3.5 years later).

Immunoblot reactivity compared with genomic detection. PCR with synovial and urine samples detected five *B. burgdorferi* sensu stricto isolates one *B. garinii* isolate, nine *B. afzelii* isolates, and one untypeable *Borrelia* isolate. Preferential reactivities were observed for serum samples from these patients by immunoblotting (to *B. burgdorferi* sensu stricto for 3 serum samples and to *B. afzelii* for 13 serum samples). The results of these two methods, performed independently, agreed with

TABLE 2. Percentage of preferential reactivity to particular *Borrelia* species by scoring method

Clinical manifestation	% Reactivity		
	Method I	Method II	Methods I and II
Neuroborreliosis (n = 31)	58	84	90
ACA (n = 31)	68	87	94
Arthritis (n = 34)	56	88	94

TABLE 3. Percentage of serum samples per disease group showing preferential reactivity for individual species by methods I and II

Clinical manifestation	% Reactivity[a]			
	B.b.ss	B.g.	B.a.	B.v.
Neuroborreliosis (n = 31)	6	52	22	10
ACA (n = 31)	7	3	84	0
Arthritis (n = 34)	62	0	29	3

[a] B.b.ss. *B. burgdorferi* sensu stricto; B.g., *B. garinii*; B.a., *B. afzelii*; B.v., *B. valaisiana*.

each other for samples from 11 patients (69%) (two samples had preferential reactivity to *B. burgdorferi* sensu stricto and nine samples had preferential reactivity to *B. afzelii*). One sample had preferential reactivity to *B. burgdorferi* sensu stricto by immunoblotting but could not be amplified with any species-specific primers. One serum sample had a preferential reactivity to *B. afzelii* by immunoblotting although the results of PCR with urine from this patient indicated an infection with *B. garinii*. Only three discrepancies were found. For two samples the strain detected in the synovial fluid was *B. afzelii*, whereas the IgG immunoblots presented a preferential reactivity to *B. burgdorferi* sensu stricto, and for the third sample, *B. burgdorferi* sensu stricto was detected in the synovial fluid by PCR, but the IgG immunoblot suggested a *B. afzelii* infection.

DISCUSSION

Our four species-specific immunoblots allowed us to observe predominant serum antibody reactivity to one *Borrelia* species in the majority of serum samples from patients with chronic LB (90% for patients with neuroborreliosis [28 of 31], 94% for patients with ACA [29 of 31], and 94% for patients with Lyme arthritis [32 of 34]). The association of a given clinical manifestation with a defined *Borrelia* species was confirmed by these results. The predominant reactivity persisted long after treatment of the disease. Indirect evidence suggested the involvement of *B. valaisiana* in some chronic clinical manifestations. Results of serological tests for LB may depend on the different isolates causing infection and on the antigenic source, because isolates from different species are rather heterogeneous (47, 48) and lead to differences in reactivities on Western blots (3, 21, 26, 27, 30, 46, 49, 50). Moreover, as the infecting species is geographic area specific (8), we studied the four observed species in Switzerland and tested patients with LB from the same area. Norman et al. (30) suggested that development, optimization, and validation of immunoblotting criteria must be done for each specific strain as different levels of reactivity to each strain may occur. Therefore, we selected the best way to determine the preferential reactivity to each *Borrelia* species. Species-specific immunoblot analysis achieved by scoring individual discriminative bands allowed determination of predominant reactivity for a large percentage of serum samples. The preferential reactivity of sera to one species was determined by a first-step analysis based on the scores for five protein bands (the 12-kDa, 16-kDa, 18-kDa, OspA, and 66-kDa proteins) with a high discriminative potential. Two of these five proteins (the 18- and 66-kDa proteins) are recommended by the Centers for Disease Control and Prevention for use in the confirmation of positive serological tests. First, the 18-kDa protein has already been described by other investigators and is now considered a good marker for LB (12, 22, 32, 38). Second, the 66-kDa protein has been described as a heat shock protein with a high degree of specificity for LB (7, 12, 25, 26). Analysis of isolates of *B. burgdorferi* sensu lato obtained in North America and Europe has demonstrated that OspA has antigenic variability and that several distinct groups can be serologically defined (45, 47). However, there are controversial reports about its specificity in immunoblots (6, 10, 12, 20). The functions of the 12- and 16-kDa antigens are not yet clear. When the rapid first-step scoring method was inconclusive, it was followed by a second-step scoring method based on the total score for the 19 selected proteins. In most instances, the analysis with 19 protein bands allowed the confirmation and extension of the initial typing, leaving only a few specimens with inconclusive reactivities. This two-step procedure presented the advantage of a first rapid scoring method, with about two-thirds of the serum samples having preferential reactivity at the end of this step and 93% having preferential reactivity at the end of the second-step. No divergence in preferential reactivities was observed between the first-step approach and the second-step approach.

A preliminary blinded study comparing typing by immunoblotting with genome detection in synovial fluids and urine from selected patients showed agreement for 69% (11 of 16) of the samples. Only 4 of 16 samples showed discrepancies, and for the remaining sample no comparison was possible. Among the four patients who provided the four samples for which discrepancies were found, one had had frequent tick bites over several years and so could have been in contact with several *B. burgdorferi* sensu lato species. Only urine was available from a second patient, and tests with the urine sample revealed the presence of *B. garinii*, whereas the serological interpretation was in favor of the presence of *B. afzelii*. A mixed infection in this patient could also be envisaged. Serum from one patient showed by IgG immunoblotting a preferential reactivity to *B. burgdorferi* sensu stricto, but no amplification with species-specific primers was possible.

The reproducibility of typing by immunoblotting was high with the sera from a given patient. Among paired serum samples from seven patients, the seven first serum samples with preferential reactivity to one of the four species were shown to have the same reactivity as the second serum samples in the pairs. Excluding the possibility of a risk of a reinfection, the preferential reactivity of a serum sample may be observed for several years. Although the possibility of reinfection caused by the bites of separate ticks cannot be excluded, the presence of several species in the lesions of EM and ACA patients may reflect the occurrence of mixed infections in the local tick populations (37). It was also shown that infection with multiple species can persist in patients for a prolonged period (11). In our study, the proportion of serum samples with undetermined reactivity was 10% for patients with neuroborreliosis, 6% for patients with ACA, and 6% for patients with Lyme arthritis. However, significantly higher scores for reactivity to two of the four *Borrelia* species were observed for two of the seven serum samples with inconclusive reactivities, suggesting mixed infections. Sera from patients with ACA clearly showed greater reactivity to *B. afzelii*, which confirmed the results described by other investigators (2, 3, 13, 42). The reactivities of sera from patients with Lyme arthritis were predominantly specific to *B. burgdorferi* sensu stricto (62%) and *B. afzelii* (29%), which has also been confirmed by other serological analyses (2, 3). However, a PCR analysis performed with small numbers of synovial fluid samples in Germany revealed no association between Lyme arthritis and *B. burgdorferi* sensu stricto (15, 43). In our study, we also observed discrepant results between the serological study (62% association with *B. burgdorferi* sensu stricto) and the PCR analysis with a small number of samples (31% *B. burgdorferi* sensu stricto). We think that the selection of patients with Lyme arthritis on the basis of their PCR results is highly dependent on the test characteristics and could have led to a biased prevalence of *Borrelia* species. In addition, in the two studies (15, 43), large proportions of samples from patients with Lyme arthritis (4 of 11 [36%] and 7 of 20 [35%], respectively) were negative by PCR.

Our results lead us to confirm an association between *B. garinii* (52% of preferential reactivity) and neuroborreliosis, although some investigators are opposed to such an association. The good match described by Eiffert et al. (14) between the distribution of *B. burgdorferi* sensu lato in ticks and CSF from patients in the same area was interpreted to occur as a result of the random selection of organisms detected in the examined ticks, but it does not take into account the predominant occurrence of nymph bites on humans (17). In Switzerland nymphs are mainly infected with *B. afzelii* (16a). The association between neuroborreliosis and *B. garinii* in Europe has already been described in other reports (2, 10, 11, 32, 42). The percentage of association in this study was nevertheless lower than that described by others. This may be explained by a falsely attributed preferential reactivity to *B. garinii* in the absence of *B. valaisiana* antigen. In this study *B. valaisiana* was tested by comparative immunoblotting for the first time, and a pathogenic potential similar to that of *B. garinii* was suggested. Moreover *B. garinii* and *B. valaisiana* have both been demonstrated to have enzootic cycles in avian hosts (18, 19, 29, 31). Furthermore, the phylogenetic position of VS116 (*B. valaisiana*) has been described as being closely related to that of *B. garinii* (5, 41). A preferential reactivity to *B. valaisiana* was observed among three serum samples from patients with neuroborreliosis and one patient with Lyme arthritis. These preliminary results provided some additional clues as to the potential pathogenicity of *B. valaisiana* in humans. As recently reported (37), *B. valaisiana* has been detected by PCR and restriction fragment length polymorphism analysis in skin biopsy specimens from two EM patients, and mixed infections that included *B. valaisiana* were identified in both EM and ACA patients. The clinical histories of the three patients who had neuroborreliosis and who were potentially infected with *B. valaisiana* were obtained. The three patients had partial facial palsy. One of them presented with EM 14 days after a tick bite.

Conclusion. Our scoring method with discriminative proteins on immunoblots allows attribution of the serum reactivity of patients with chronic Lyme borreliosis to one *Borrelia* species for most samples. Our results suggest an organotropism of *Borrelia* species and provide some evidence of the pathogenic potential of *B. valaisiana* in humans.

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